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In vitro photodynamic inactivation of *Candida albicans* by phenothiazine dye (new methylene blue) and Indocyanine green (EmunDo®)

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KEYWORDS
Antifungal photodynamic therapy; Photothermal therapy, *Candida albicans*

Summary
Background: The application of a new generation of photosensitizers to increase the efficacy of antifungal photodynamic therapy (aPDT) is an important aspect of PDT. Thus, this *in vitro* study is aimed to evaluate the antifungal efficacy of the photo-elimination of *Candida albicans* with photothermal and antifungal photodynamic therapy.
Method and material: aPDT with new methylene blue and photothermal therapy with EmunDo® were applied to a fungal suspension, which was then subcultured in Sabouraud dextrose agar (SDA). The *C. albicans* colonies were counted and are expressed as colony-forming unit per milliliter (CFU/ml).
Results: aPDT with either EmunDo® or new methylene blue (NMB) considerably diminished the viability of inoculated *C. albicans* ($P < 0.001$) by log reduction of 1.9 and 3.37, respectively, compared with the control group respectively, compared with the control group. The antifungal potency or dark toxicity of the two photosensitizers alone did not significantly differ ($P = 0.70$). The same trend was observed for the light sources (λ: 810 nm vs. λ: 630 nm), which also did not significantly differ ($P = 0.78$).
Conclusion: The photo-elimination of *C. albicans* with either new methylene blue or EmunDo® as a photosensitizer can reduce the viability of fungal cells. Although the result of this study is encouraging, further investigations are warranted to determine clear protocols for the reliable and safe application of this method in clinical practice.

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Introduction

Oral candidiasis is the most common opportunistic infection that influences the oral mucosa. The most predominant etiological agent in oral candidiasis is *Candida albicans*, which corresponds to 70—80% of all microorganisms isolated from oral lesions [1,2]. Oral candidiasis is a common manifestation of fungal infections, especially in immunocompromised patients, such as HIV/AIDS patients and cancer patients receiving radiotherapy and chemotherapy [3]. This microorganism does not cause systemic or local problems in healthy patients. However, infections systematically or locally emerge when the natural balance between the microorganism and immune system is disrupted [4]. Numerous chemotherapeutic drugs in the form of topical and systemic antifungal drugs are administered to treat fungal infections [5]. The emergence of drug resistant forms of candidiasis, especially in HIV/AIDS patients, is a growing concern among clinicians [6]. Fungal resistance can increase the prevalence of the disease. Moreover, the side effects of these drugs, such as interactions with other drugs and hepatotoxicity, limit their use. Long periods of drug therapy and patient incompliance are probable in some cases [7,8]. Thus, research has primarily focused on novel therapeutic approaches to overcome these complications and to increase the efficacy of treatment [9,10].

Photodynamic therapy has primarily been successfully used to treat malignant disease [11,12]. However, it has also been indicated and deployed to treat bacterial, fungal and viral infections in recent years. Antifungal photodynamic therapy is a developing area of research and has attracted a great deal of attention in the treatment of fungal infections [13—15].

Photodynamic therapy utilizes the administration of a light source of specific wavelength and a photosensitizer (PS) that accumulates in the target cell and is activated by a light source in the presence of oxygen. The resulting reaction induces cell death due to the production of toxic oxygen species, such as singlet oxygen and or free radicals [16,17]. These reactions occur in a limited space; thus, this type of local therapy has additional benefits compared with systemic therapy [16,17]. Among photosensitizers, phenothiazinium derivatives, including methylene blue and toluidine blue, are most commonly used to photodynamically inactivate microorganisms primarily due to their low toxicity and demonstrated good outcomes in the inactivation of *C. albicans* [18,19].

The application of a new generation of photosensitizers to increase the efficacy of anti-fungal photodynamic therapy is an important aspect of aPDT. Thus, promotion of novel PS agents which allows to use lower doses of PS and a more selective reaction is important in photodynamic reaction [20].

When the oxygen content is low, the efficacy of aPDT may decrease due to dependency of this process on oxygen. Binding PS to the target cell and killing fungal cells *via* light activation increases the temperature of PS, but photothermal therapy can overcome the problem of oxygen deficiency. Hence, photothermal therapy, which is an oxygen-dependent process, could have additional benefits due to its hyper-thermic antifungal mechanism of action [21].

The susceptibility of fungal microorganisms to photodynamic therapy has been demonstrated *in vitro* and *in vivo* [22—24]. However, conclusive evidence to confirm a clear and precise protocol for the photodynamic destruction of fungal pathogens is lacking, and further studies are required.

The aim of this *in vitro* study was to compare the effectiveness of the photoelimination of *C. albicans via* two aPDT methods (new methylene blue and EmunDo®).

Methods and materials

Microorganism preparation

A suspension of *C. albicans* (PFCC 5027) with an optical density of 0.5 McFarland and 10^6 colony forming unit per milliliter (CFU ml^{-1}) was prepared at Tarbiat Modares University, Tehran, Iran.

Photosensitizer

New methylene blue N zinc chloride double salt (NMBN, Sigma—Aldrich, Germany) (Fig. 1) was purchased, and a 10 μM stock solution of NMB was prepared by dissolving it in phosphate buffered saline (PBS). This stock solution was diluted 4-fold in the experiments. It was stored at 4 °C in the dark until use.

EmunDo® solution (A.R.C. laser GmbH, Nurnberg, Germany) (Fig. 2) was prepared according to the manufacturer's instructions at a concentrations of 1 mg/ml and stored in the dark.

Light sources

The light source used to activate NMB was a InGaAlP red light laser (MUSTANG, Russia) source, and the samples were

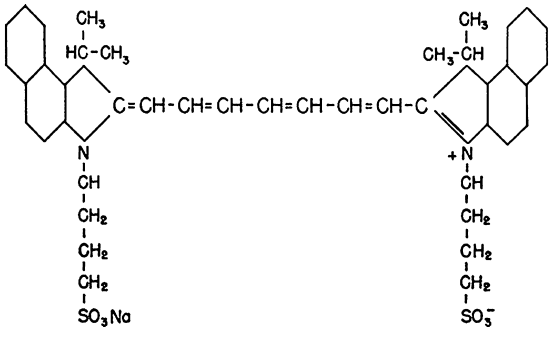

Figure 1 Chemical structure of new methylene blue used in the study.

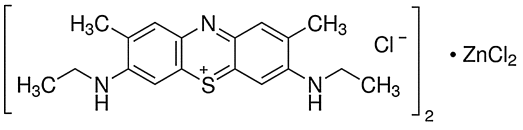

Figure 2 chemical structure of Indocyanin green.

Table 1 Effect of treatments in each group of study on the viability of *Candida albicans* (log-transformed mean CFU/ml).

	IRLE	IRL	E	CO	RLN	N	RL
Count (CFU/ml, E5[a])	0.7 (0.48)b,d	1.7 (0.48)a,d,e	1.4 (0.51)d,e	0.03 a,c,e,f,g	0.3 (0.49)b,c,d,g	2.33 (0.19)d	2.50 (0.18)d,e

The letters indicate the presence of statistically significant differences for the mentioned group (Games–Howell test: $P < 0.05$).
[a] E5 indicates that presented data should be multiplied by 10^5. Red Laser and NMB (RLN), Infrared Laser and EmunDo® (IRLE), Red Laser (RL), Infrared Laser (IRL), NMB (N), EmunDo® (E), no light, no photosensitizer (CO).

irradiated for 12.5 min with an output power of 10 mW, emission peak at 630 nm, spot area of 0.5 cm^2 and resultant energy density of 15 J/cm^2.aPDT with EmunDo® was combined with a diode laser at a wavelength of 810 nm (A.R.C. laser GmbH, Nurnberg, Germany). According to the manufacturer's recommendations, the following settings were used: 300 mW, continuous mode, 30 s and resultant energy density of 55 J/cm^2, spot size of 4.5 mm to ensure to cover the whole surface area of each well, three times of irradiation in three isolated part of well are carried out.

aPDT with NMB and EmunDo®

Aliquots of 100 μl of PS, fungal suspension or PBS solution were added to the 12 predefined wells of a 96-well flat-bottomed micro-titer plate according the specified treatment groups. The plates were shaken well to ensure that the suspension was well mixed. For the NMB group, a mixture of NMB and fungal suspension was stored in the dark at 28 C for 30 min and 5 min for EmunDo® prior to laser irradiation. This process ensured sufficient time to allow for the satisfactory absorption of photosensitizer by fungal cell walls. The experimental and control groups were classified as the follows:

1. Experimental aPDT: Red Laser and NMB (RLN)
2. Experimental aPDT: Infrared Laser and EmunDo® (IRLE)
3. Positive control: Red Laser (RL)
4. Positive control: Infrared Laser (IRL)
5. Positive control: NMB (N)
6. Positive control: EmunDo® (E)
7. Negative control: no light, no photosensitizer (CO).

The final concentrations of fungal suspension, NMBN and EmunDo® were 0.5×10^{-5}, 2.5 μM and 50 μg/ml, respectively. All procedures were carried out in a laminar-flow hood in aseptic conditions, and the vertical distance between the light device and well surface were fixed in all processes with a microphone holder to assure the accuracy of the desired laser energy. Only 12 wells were selected to avoid the diffusion of laser light to the adjacent wells.

After exposure, the suspensions were removed and serially diluted in phosphate buffered saline (PBS) to give dilutions of 10^{-2}–10^{-4} times the original concentration. Finally, 10^3 μl of the diluted solution was added to the Sabouraud dextrose agar (SDA) medium in petri dishes. Each test consisted of ten replicates. After incubation in the dark at 37 °C for 48 h, the number of colony-forming units per milliliter was counted by a blind examiner.

Continuous data are expressed as the means (standard deviation). The data were analyzed using a one-way analysis of variance (ANOVA) and Games–Howell test for further multiple comparisons due to a significant Levene test. A two-tailed *P*-value less than 0.05 was considered as statistically significant.

Results

Significantly different colony counts were observed between the groups involved ($F(6) = 17.19$, $P < 0.001$) (Table 1). The colony count of the control group (CO), which was treated with neither laser irradiation nor photosensitizer, was considerably higher those of the other groups ($P < 0.05$). aPDT with either EmunDo® or new methylene blue (NMB) considerably diminished the viability of inoculated *C. albicans* ($P < 0.001$) by log reduction of 1.9 and 3.37 compared with the control group (Fig. 3). The difference between the potency of the photosensitizer alone E (EmunDo®) and N (NMB) did not reach statistical significance ($P = 0.70$). The same trend was observed for the light sources (IRL: 810 nm vs. RL: 630 nm); their effects did not significantly differ ($P = 0.78$). The CFU reduction was most pronounced in the groups that were both sensitized and treated with a laser. Two PDT groups (IRLE, 810 nm and RLN, 630 nm) equally reduced the CFU counts ($P = 0.59$).

Discussion

In this *in vitro* study, we demonstrated the efficacy of the photo-inactivation of *C. albicans* by two new photosensitizing agents, new methylene blue (NMB) and EmunDo®. The photo-inactivation by both of these photosensitizers significantly reduced the viability of *C. albicans* by log reduction of 1.9 and 3.37 compared with control group ($P < 0.001$).

Previous generations of phenothiazine agents, such as MB and TBO, have been conventionally used in aPDT against both bacterial and fungal microorganisms [18,19]. Despite their fungicidal effect, some authors reported that these phenothiazine dyes are not optimal fungicides or their effective killing doses for *C. albicans* are considerably high [25,26]. Hence, examining new derivatives of phenothiazine to increase the efficacy of aPDT is of crucial importance. To our knowledge, a limited number of studies have investigated the efficacy of NMB against *C. albicans*. Wainwright et al. investigated the photo-inactivation of *C. albicans* with NMB and showed 1.74-log reduction of *C. albicans* in response to irradiation with a diode laser at a wavelength of 631 nm and energy density of 15 J/cm^2. The results of this study agree with this previous study (a 93% reduction in the

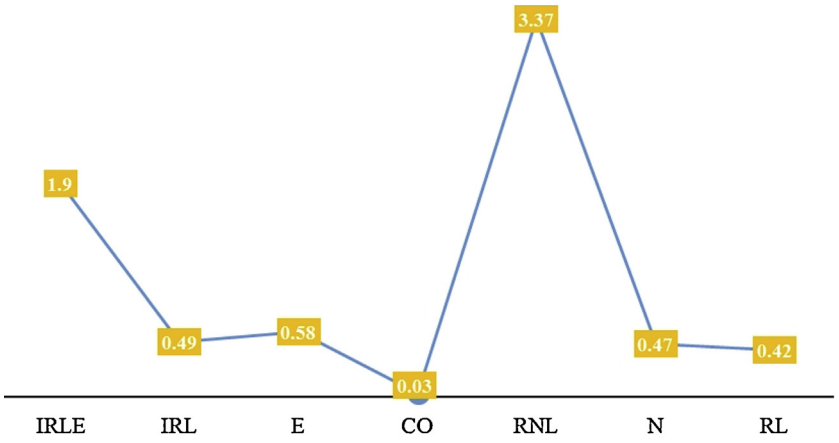

Figure 3 Effect of treatments in each group of study on the viability of *Candida albicans*. Log reduction of colony forming unit per milliliter (CFU ml^{-1}) separated for the applied photosensitizer and corresponding laser source compared with control group. Red Laser and NMB (RLN), Infrared Laser and EmunDo® (IRLE), Red Laser (RL), Infrared Laser (IRL), f:NMB (N), c:EmunDo® (E), d:no light, no photosensitizer (CO).

number of *C. albicans* colonies based on CFU/ml), although the light source in their study was LED. Furthermore, exposure to light or NMB alone did not appear to significantly affect *C. albicans*, which is similar to the findings of this study [26].

We also studied EmunDo®, which has recently been popularized in the field of dentistry. According to the manufacturer's statement, EmunDo® includes mainly Indocyanine green (ICG), which is a water-soluble tri-carbocyanine dye with high affinity to the near infrared region ($\lambda = 810$ nm). ICG is approved by the US food and drug administration for medical diagnostic studies, such as visualizing retinal and choroidal vasculatures [27,28]. The high absorption of light in the infrared region at 805 nm increases the penetration depth compared with many other photosensitizers [29,30]. ICG binds to the plasma protein and does not undergo chemical changes in the body and rapidly excreted to the bile duct. It has already been shown that this dye has acceptable properties to act as a photosensitizer [29,31,32]. Its antifungal killing mechanism is not precisely understood, but both photochemical and photodynamic reactions have been implicated as a possible mechanism of action. While Gomes et al. observed that cells were photo-damaged *via* free radical formation after incubation with ICG [33], photothermolysis is preponderant in killing sebaceous glands in the treatment of acne [34]. Nagahara et al. observed a significant reduction of *Porphyromonas gingivalis* and a temperature increase of at least 4.23 °C in 1 min after incubation with ICG-loaded nanospheres with an 805 nm diode laser [28]. Fekrazad et al. compared the efficacy of photothermal therapy with EmunDo® and PDT with Toluidine blue O or Radachlorin against a standard suspension of *Streptococcus mutans* and observed a significant reduction of *S. mutans* after treatment with either approach and no significant difference between the groups. They demonstrated for the first time that the efficacy of photo-elimination therapy with EmunDo® in reducing the number of *S. mutans* colonies was at least as effective as those of the other two dyes [21].

To our knowledge, no study has focused on utilizing the ICG-mediated photo-inactivation of *C. albicans*. Thus, the data obtained for aPDT with ICG cannot be compared with other studies. The significant reduction in the number of *C. albicans* after photo-inactivation with EmunDo®, which was similar to treatment with aPDT and NMB, may reflect the potential efficacy of ICG in the photodynamic or photothermal inactivation of *C. albicans*. However, the precise mechanism of action of ICG is not clear. Nevertheless, a potential photothermal mechanism could present additional benefits over PDT, especially in low oxygen areas, such as anaerobic infections and deep periodontal pockets, where oxygen is limited. Furthermore, since ICG is excited in higher wavelength compared with NMB dye, its ability to penetrate deeper in the tissue is higher than NMB. So presumably its antimicrobial application in deeper infected site is reinforced [35]. Oxygen has clearly been described as a crucial substrate for photodynamic-derived antibacterial action. Notably, a wavelength of fox Q810.plus (810 nm) is applicable in other procedures in dental practice, such as bleaching, low level laser therapy and soft tissue surgery.

The application of EmunDo® or NMB alone did not significantly affect *C. albicans*. This finding indicated that the dark toxicity of ICG and NMB at low concentration (2.5 μM) is not influential without the application of a laser source of appropriate wavelength. Wainwright et al. showed that the minimum inhibitory concentration for the dark toxicity of NMB against *C. albicans* was 25 μM, and the dark toxicity against *C. albicans* was insignificant below this does. This finding agreed with our study, which utilized 2.5 μM NMB [26].

Although the dark toxicities of both lead dyes were insignificant in our study, both exerted some toxic effects on *C. albicans* cells. The possible effects of both dyes on natural host cells are important to note, especially at high concentrations. As mentioned above, Wainwright et al. reported that a low concentration of NMB (1 or 2.5 μM) was only slightly toxic to mammalian cells, but this effect was amplified at high concentrations (10 μM) [26]. Dai et al. assessed the efficacy of PDT with high concentration of NMB; CFU inactivation was observed at 20 μM of NMB, a wavelength of 635 ± 15 nm and a fluency of 75 J/cm^2 for 5 min and 4.43

log. They speculated that the improved efficacy of NMB compared with MB and TBO is likely due to more lipophilic nature of NMB, while MB and TBO are hydrophilic [36]. These authors did not examine high concentrations; they mentioned that a high concentration of NMB exerted better fungicidal effects than low concentrations. Thus, the precise minimum inhibitory effect or optimal therapeutic dose must be determined while considering safety precautions. This information is of crucial importance to treat resistant forms of candidiasis, which may require high concentrations of photosensitizing agents.

The photoactivation of EmunDo® with a diode laser with specific exposure parameters (300 mW, continuous, 30 s and energy density of 24 J/cm^2) allowed for a shorter irradiation time compared with the activation of NMB with a red light laser, which required an irradiation time of 12.5 min with an output power of 10 mW and energy density of 15 J/cm^2. Thus, an increased output power and decreased exposure time are desirable for therapeutic targets and the selection of laser system.

While only a limited number of studies have demonstrated the efficacy of NMB as a novel photosensitizer, one should note that the exposure parameters, such as the power, irradiation time, and energy density and photosensitizer concentration, differed among these studies, which hinders comparisons between these studies.

In conclusion, the photo-elimination of *C. albicans* with either new methylene blue or EmunDo® as a photosensitizer can reduce the viability of fungal cells. Although the result of this study is encouraging, further investigations are warranted to determine the protocols to reliably and safely apply this method in clinical practice.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Acknowledgments

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